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Development of a Novel Chemical Probe for the Selective Enrichment of Phosphorylated Serine- and Threonine-Containing Peptides

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Gaining insight into phosphoproteomes is of the utmost importance for understanding regulation processes such as signal transduction and cellular differentiation. While the identification of phosphotyrosine-containing amino acid sequences in peptides and proteins is now becoming possible, mainly because of the availability of high-affinity antibodies, no general and robust methodology allowing the selective enrichment and analysis of serine- and threonine-phosphorylated proteins and peptides is presently available. The method presented here involves chemical modification of phosphorylated serine or threonine residues and their subsequent derivatization with the aid of a multifunctional probe molecule. The designed probe contains four parts: a reactive group that is used to bind specifically to the modified phosphopeptide, an optional part in which heavy isotopes can be incorporated, an acid-labile linker, and an affinity tag for the selective enrichment of modified phosphopeptides from complex mixtures. The acid-cleavable linker allows full recovery from the affinity-purified material and removal of the affinity tag prior to MS analysis. The preparation of a representative probe molecule containing a biotin affinity tag and its applicability in phosphoproteome analysis is shown in a number of well-defined model systems of increasing degrees of complexity. Amounts of phosphopeptide as low as 1 nmol can be modified and enriched from a mixture of peptides. During the development of the β-elimination/nucleophilic addition protocol, special attention was paid to the different experimental parameters that might affect the chemical-modification steps carried out on phosphorylated residues.

Introduction

The reversible phosphorylation of serine, threonine, and tyrosine residues in peptides and proteins is generally recognized to play a pivotal role in the regulation of virtually all cellular functions. With an estimated one third of all eukaryotic proteins being amenable to this form of post-translational modification, phosphorylation is widely acknowledged as a regulating mechanism touching on almost every known signaling pathway.[1] In spite of its importance, the global analysis of phosphoproteomes remains a challenge that is far from being met. Often encountered problems involve heterogeneous phosphorylation patterns within a given protein and their relatively low abundance, necessitating the development of selective enrichment techniques. In general, there is a clear need for robust analytical methodologies that allow the quantitative mapping of phosphoproteins and their phosphorylated sites and rapid screening for deviant phosphorylation events.

Several approaches towards quantitative phosphoproteomics have been described in the literature[2–5] and, as in many fields in proteomics, mass spectrometric analysis plays a crucial role in these studies.[5–10] While analysis of phosphorylated tyrosyl residues (Tyr(P)) in peptides and proteins is facilitated by the commercial availability of specific antibodies,[11–13] the use of immunochemical techniques for the enrichment and detection of proteins containing Ser(P) and Thr(P) (accounting for $>99\%$ of all phosphorylated species) has only rarely been demonstrated.[8,14] For tackling analytical problems associated with these two types of phosphorylated amino acid residues, different strategies based on affinity chromatographic enrichment of phosphopeptides from tryptic protein digests, followed by mass spectrometric analysis have been reported.[15–22] Nevertheless, because of the poor ionization efficiencies of phosphopeptides in the positive ionization mode, probably resulting from proton sequestration by the acidic phosphate group, methods in which the phosphate ester is chemically modified prior to MS might be expected to be more sensitive.[9] So far, a

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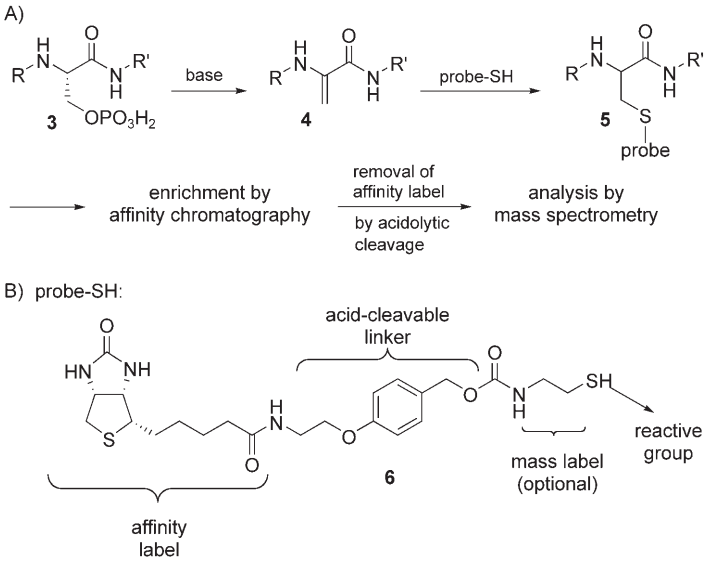
number of methods for phosphoprotein and -peptide analysis and/or enrichment based on chemical modification have been described.[15,23–31] However, some of these approaches suffer from certain limitations, such as relatively low reaction efficiency and, as a result, low yields, which hamper proteomics applications. We chose a stepwise development of a chemical modification/multifunctional probe addition strategy in investigations geared towards general methods for assessing phosphoserine/phosphothreonine-containing (phospho)proteomes. During this process we focused on improvement in the efficiency of the individual reactions and a stepwise extrapolation of the results obtained from the test conditions.

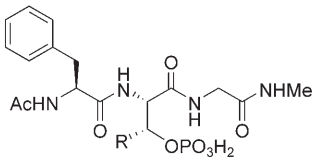


Scheme 1. A) Base induced β-elimination followed by Michael addition of an SH-containing probe. B) Thiol-containing multifunctional probe.

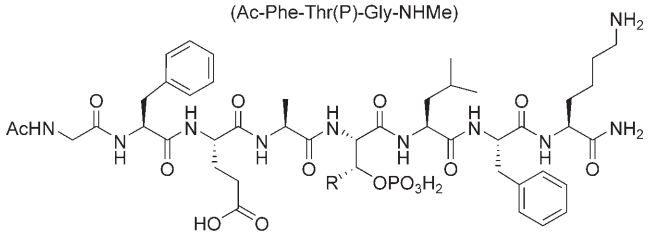
Results and Discussion

Approach

The following test systems, of increasing degrees of complexity, were developed for evaluation of our labeling protocol and analysis of phosphopeptides:



1a: R = H, Ac-FpSG-NH_2
(Ac-Phe-Ser(P)-Gly-NHMe)
1b: R = Me, Ac-FpTG-NH_2
(Ac-Phe-Thr(P)-Gly-NHMe)



2a: R = H, Ac-GFEApSLFK-NH_2
(Ac-Gly-Phe-Glu-Ala-Ser(P)-Leu-Phe-Lys-NH_2)
2b: R = Me (Ac-GFEApTLFK-NH_2)
(Ac-Gly-Phe-Glu-Ala-Thr(P)-Leu-Phe-Lys-NH_2)

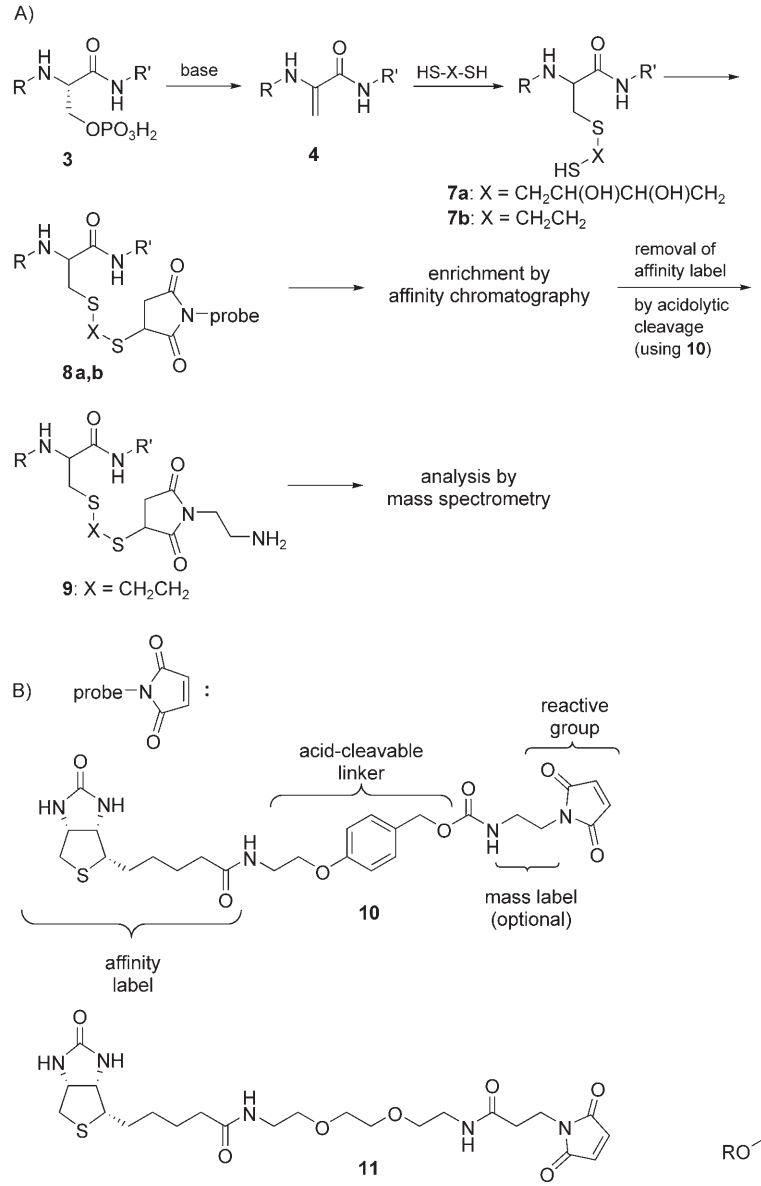
phosphorylated tripeptides **1a** and **1b**, phosphorylated octapeptides **2a** and **2b**, and a mixture of peptide **2a** and a number of other, nonphosphorylated, peptides. This mixture was viewed as a model for protein tryptic digests, and served to assess affinity enrichment after probe attachment.

Our approach for studying Ser(P) and Thr(P) residues in peptides and proteins exploits the well studied base-promoted β-elimination reaction of aliphatic phosphate esters (**3**) in basic media[24,25,29,32] (Scheme 1A). This step involves the creation of a Michael acceptor moiety (**4**) that is susceptible to attack by nucleophiles such as thiols, leading to adduct **5**. We therefore developed multifunctional probe molecule **6** (Scheme 1B) that consists of four functional modules: 1) a nucleophilic reactive site (thiol), 2) a part in which heavy isotopes may be incorporated (in the ethylene chain), 3) an acid-labile "Wang"-type linker, and 4) a biotin moiety that allows affinity-based purification.

Incubation of **6** with β-eliminated peptides derived from, for example, **2a** or **2b** was expected to result in the formation of a covalent adduct **5** through Michael addition of the sulfhydryl moiety of **6** to the α,β-unsaturated amino acid residue in the peptide (Scheme 1A). However, no covalent linkage of the probe to the peptide was observed, even upon addition of a tenfold excess of **6** to a solution of a β-eliminated peptide derived from **2a**. The size of the nucleophile was probably detrimental to its reactivity and a smaller thiol nucleophile—to be used in high excess—-might be more advantageous. This enticed us to consider alternative strategies.

A possible strategy involves the addition of a dithiol to β-eliminated phosphopeptides **4** (Scheme 2A). The originally phosphorylated serine or threonine residue is thus functionalized with a thiol group (**7**), which serves as a handle for linking of the peptide to a probe molecule. A similar successful approach has been reported in the literature.[25,27,29,33] However, the conditions described in the literature were not successful in our hands for modification of phosphopeptides **2a** and **2b**, so an optimized procedure for β-elimination and subsequent ethanedithiol (EDT) addition was developed. In addition to supplying a handle for probe attachment, the generation of a thiol-functionalized residue also allows the application of available cysteine-labeling methods. Unlike **6**, the new probe (**10**) carried an electrophilic group, since peptide **7** is now functionalized with a sulfhydryl group. This probe (Scheme 2B) again included a biotin affinity label, a Wang-type linker, but also the electrophilic maleimide functionality, incorporated for chemoselective reaction with the generated thiols on the originally phosphorylated amino acid residues.

A similar, commercially available, probe that lacks the acid-sensitive part (**11**; see Scheme 2B) had been used earlier by



Scheme 2. Dithiol modification of phosphopeptides and electrophilic addition of the probe.

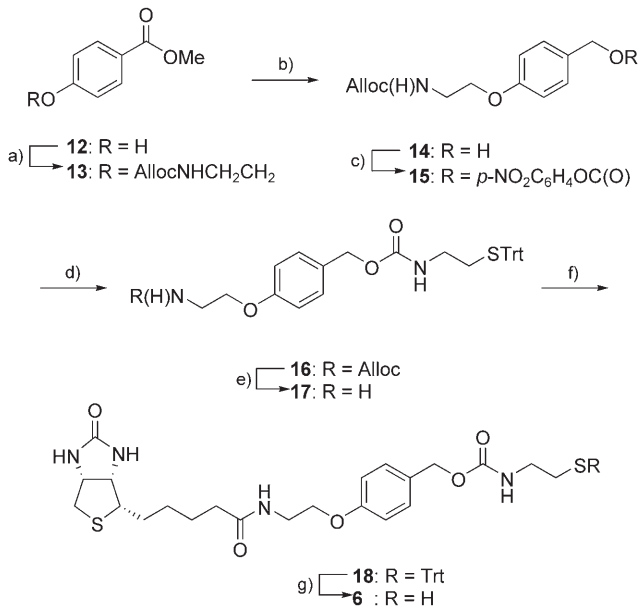
Oda et al. for isolating a phosphorylated protein that was used to spike a complex mixture.[27] Other methods derived from this strategy have also been described.[33,34] However, the presence of the cleavable linker in our molecule offers means to circumvent several of the shortcomings of the technique, such as incomplete recovery from the (strept)avidin material used during the affinity-based isolation/enrichment. In addition, incorporation of the acid-labile linker in **10** affords small, stable adducts—that no longer include the biotin moiety—with the previously phosphorylated serine and threonine residues, thus leading to more easy interpretable mass spectra.

In the first step, the validity of these assumptions was tested with well-defined model systems. Thus, two model octapeptides, **2a** and **2b**, containing either a Ser(P) or a Thr(P) residue, were used in experiments in which the β-elimination/Michael addition/probe addition sequence and avidin affinity chromatography were applied to isolate and enrich both peptides from peptide mixtures of known compositions. In addition, since no systematic optimization studies of the β-elimination and Michael addition reactions could be found in the literature, and as existing reports in general deal exclusively with the reactivity of Ser(P)-containing peptides, two phosphorylated model tripeptides, **1a** and **1b**, were prepared with the aim of studying experimental parameters affecting these transformations for both Ser(P) and Thr(P) residue-containing peptides.

Synthesis of the probe molecules

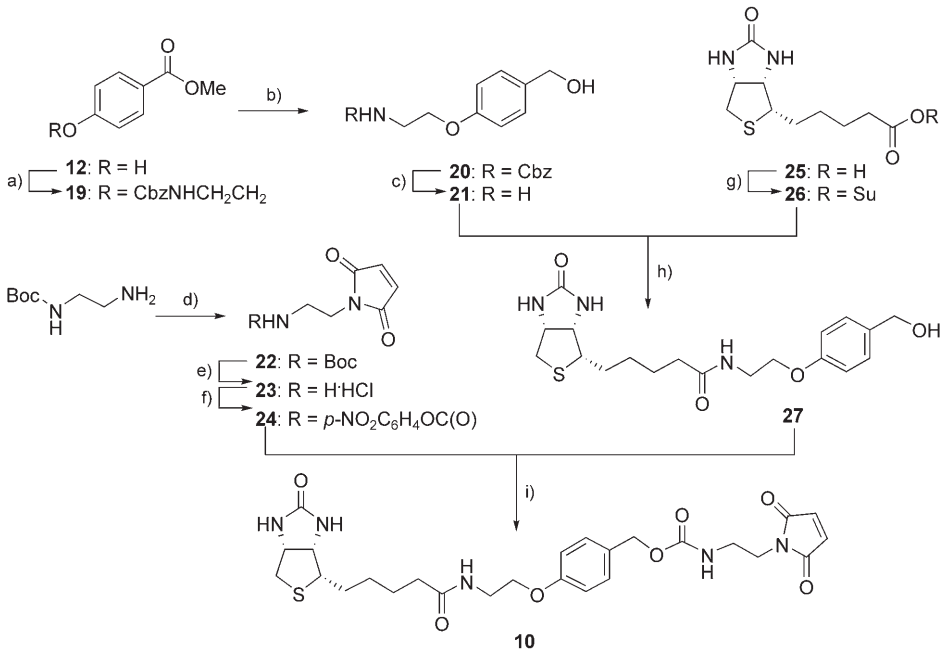
The synthesis of thiol-functionalized biotin probe **6** (Scheme 3) began with the alkylation of methyl 4-hydroxybenzoate (**12**) with *N*-Alloc-2-bromoethylamine. The alkylation product **13** was reduced with $LiAlH_4$ to give benzylic alcohol **14**, which was subsequently converted into activated carbonate **15** by treatment with *p*-nitrophenyl chloroformate. Treatment with *S*-tritylcysteamine in the presence of Et_3N then furnished carbamate **16**. After Alloc deprotection, the resulting free amine (**17**) was used in a BOP-mediated coupling to biotin, affording the *S*-protected probe **18**. The free probe **6** was generated in situ when required for labeling studies.

For the maleimide-containing probe (**10**), the acid-cleavable linker moiety **21** was synthesized from methyl 4-hydroxybenzoate in a sequence consisting of alkylation, $LiAlH_4$-mediated reduction of the ester



Scheme 3. Synthesis of probe molecule **6**. Reagents and conditions: a) *N*-Alloc-2-bromoethylamine, K_2CO_3, DMF, 81%. b) $LiAlH_4$, Et_2O, 91%. c) *p*-Nitrophenyl chloroformate, pyridine, CH_2Cl_2, 79%. d) *S*-Tritylcysteamine, Et_3N, THF, 64%. e) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 89%. f) Biotin, BOP, Et_3N, DMF, 93%. g) TFA, EtSH, CH_2Cl_2, quant.

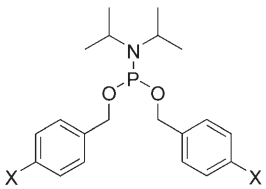
functionality, and removal of the benzyloxycarbonyl protecting group (Scheme 4). Building block **24**, containing the maleimide group and a site in which stable isotopes can, optionally, be incorporated, was obtained from the addition of maleic anhydride to monoprotected ethylenediamine and subsequent ring closure, with acidolytic cleavage of the Boc protecting group from **22** and the addition of 4-nitrophenyl chloroformate to the resulting amine **23** leading to **24**. For assembly of the probe molecule, the "Wang"-derived linker was coupled to D-biotin *N*-hydroxysuccinimidyl ester **26**, prepared by the method of Bayer and Wilchek.[35] The complete probe molecule (**10**) was obtained in good yield after carbamoylation of benzyl alcohol **27** with 4-nitrophenyl carbamate **24**.



Scheme 4. Synthesis of probe molecule **10**. Reagents and conditions: a) *N*-Cbz-2-bromoethylamine, K_2CO_3, μW, neat, 83%. b) $LiAlH_4$, Et_2O, 94%. c) H_2, Pd/C, 98%. d) Maleic anhydride, Et_3N, Et_2O; then BOP, Et_3N, CH_2Cl_2, 38%. e) HCl, Et_2O, 96%. f) *p*-Nitrophenyl chloroformate, Et_3N, CH_2Cl_2, −18 °C, 37%. g) DCC, DMF, 90 °C, 99%. h) **21**, **26**, DMF, 85%. i) **24**, **27**, Et_3N, DMF, 37%.

Model tripeptides

Tripeptides Ac-Phe-Ser(P)-Gly-NHMe (**1a**) and Ac-Phe-Thr(P)-Gly-NHMe (**1b**) were prepared by standard *tert*-butyloxycarbonyl (Boc) solution-phase peptide synthesis. The amino termini of the peptides were capped with acetyl groups by treatment with *N*-hydroxysuccinimidyl acetate (AcOSu) and the seryl or threonyl residues were phosphorylated with reagent **28a**, prepared as described previously.[36–38]



The 4-chlorobenzyl protecting groups were removed by Pd-catalyzed hydrogenolysis, and phosphopeptides **1a** and **1b** were purified by reversed-phase column chromatography (C-8). These compounds were prepared for optimization of the β-elimination and dithiol addition reaction on a preparative scale (10–100 mg), allowing thorough characterization of all products formed. For the optimization of the β-elimination reaction, peptides were incubated at 45 °C with different inorganic (NaOH, CsOH, $Ba(OH)_2$) and organic bases (DBU, $NaOCH_3$, Et_3N) in MeCN/H_2O mixtures (60:40 to 90:10 *v/v*). For every solvent mixture tested, experiments were run with two, five, seven, and ten molar equivalents of base. In all cases, nearly quantitative elimination of phosphate from the Ser(P)-containing peptide could be achieved within 3 h. Formation of the dehydrobutyrine residue in the phosphothreonine-containing peptide turned out to be significantly slower, however, requiring up to 6 h of incubation: hydrolysis of the phosphate ester could be detected in the case of the alkali bases and was most pronounced in experiments with NaOH. The product of this competing side reaction, Ac-Phe-Thr-Gly-NHMe, could be isolated in yields of up to 10%. Although, from these results, none of the used bases was found to be a significantly superior promoter of the β-elimination reaction, we decided to use $Ba(OH)_2$ in all further experiments. At this point it should be noted that peptides resulting from other post-translational modifications on serine and threonine, such as glycosidation[39] and the less often encountered sulfonation,[40] may also undergo β-elimination under these conditions. No reliable procedure for *selective* β-elimination of phosphorylated Ser/Thr residues in the presence of glycosylated Ser/Thr residues has been reported to date.

Although several authors claim that the use of elimination mixtures containing the highly polar aprotic DMSO gave favorable results in terms of the kinetics and yields of the reaction,[29,33] this observation could not be reproduced with our model tripeptides. Comparable results were obtained when the elimination reaction was run in MeCN/H_2O or in a standard elimination mixture containing DMSO, EtOH, and H_2O (3:1:1). Furthermore, because of the poor compatibility of DMSO with the process of matrix crystal formation (MALDI) and electrospray ionization, solvent systems based on MeCN/H_2O mixtures were considered to be more suitable for this application.

After isolation of the dephosphorylated peptides, optimization of the dithiol addition reaction was performed by incubation with two, four, or six equivalents of 1,4-dithiothreitol (DTT) and a catalytic amount of base (Et_3N, 0.2 equiv.). In all cases, DTT addition proceeded in a quantitative fashion within 3 h.

Again, no additional advantages in terms of yields and kinetics were observed with the DMSO/EtOH/H_2O mixture relative to MeCN/H_2O systems.

Model octapeptides

Octapeptides **2a** and **2b** were synthesized by standard 9-fluorenylmethoxy-carbonyl (Fmoc) solid-phase synthesis on a cross-linked polystyrene resin with a polyethylene glycol spacer and the Rink amide linker (*p*-{(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl}-phenoxyacetic acid). After Fmoc deprotection of the *N*-terminal glycine residues, the amino termini of the peptides were capped on the solid phase by treatment with *N*-hydroxysuccinimidyl acetate (AcOSu). Phosphorylation was carried out with reagent **28a** followed by oxidation with *t*BuOOH in the case of the serine-containing peptide and with reagent **28b** in that of the threonine peptide.[36,37,41] The peptides were then cleaved from the resin by acidolysis; this step simultaneously removes the phosphate benzyl esters and side chain protecting groups present on lysine and glutamic acid residues. Final products were lyophilized and used as such. Initially, experiments were carried out in order to establish whether the phosphopeptide in amounts of 10 pmol to 10 nmol could be isolated from solution by the series of transformations described earlier. Conditions optimized for the β-elimination and dithiol addition of model tripeptides were applied: MeCN/H_2O (90:10 *v*/*v*) was chosen as the solvent and $Ba(OH)_2$ as the base. The β-elimination process was found to be similarly straightforward, but was invariantly accompanied by some hydrolysis of the phosphate ester, in the cases of both the Ser(P)- and the Thr(P)-containing peptide. Addition of DTT, however, proved to become substantially more problematic on downscaling: even with 50 equivalents, only a minor fraction of the dehydropeptides could be transformed into the corresponding dithiol adducts. Since the introduction of even larger excesses of DTT was expected to compromise the subsequent process of probe binding, due to scavenging of maleimide by unreacted dithiol, we decided to use volatile ethanedithiol (EDT) in all further experiments. Unreacted portions of this reagent could then be removed from the system by evaporation prior to probe addition. However, large excesses (~10^4 equivalents) of both EDT and base (Et_3N) were required to achieve near-quantitative dithiol addition. This required excess resulted in the formation of unexpected byproducts when MeCN was used as solvent. In addition to the expected peaks in the MS spectrum (e.g., dehydropeptide, EDT adduct), several other peaks were observed, all displaying a +41 Da mass shift with respect to the expected peaks. MS/MS analysis revealed that these peaks had arisen from addition of MeCN to the lysine residue. We suspect that high concentrations of EDT and base in MeCN result in the formation of a thioacetimidate, which would be susceptible to attack by nucleophiles such as the lysine residue ε-amino group.

This observation led us to change the solvent of EDT addition (and β-elimination) to MeOH. Although this requires somewhat longer reaction times, β-elimination and EDT addition proceeded smoothly, and no unexpected side products were observed.

The phosphorylated peptides **2a** and **2b** were subjected to the β-elimination/EDT addition protocol and were subsequently affinity purified with immobilized avidin. After cleavage of the Wang-type linker, the supernatant was analyzed by MALDI-TOF mass spectrometry, which showed that the originally phosphorylated serine- and threonine-containing peptides had been modified. The subsequent mass spectrometric fragmentation of the modified peptides is shown in Figure 1. The resulting MS/MS spectrum not only contains nearly complete series of b and y ions, which can be used for sequence tagging of the peptide and determination of the site of phosphorylation, but also shows the modified immonium ion at *m*/*z* 276.1 (in the case of the serine-modified peptide) and *m*/*z* 290.1 (in that of the threonine-modified peptide), which show that the phosphorylated amino acid residue has indeed been modified in the desired way by the protocol used. These specific immonium ions may potentially be used as marker ions for the phosphorylated serine and threonine peptides. In comparison with fragmentation of a phosphorylated peptide, which normally mainly results in loss of the labile phosphate group and requires further (MS/MS/MS) fragmentation to provide sequence information, the approach shown here has the advantage that most of the label stays on the amino acid residue, thereby allowing sequence tagging and phosphorylation site determination. In addition, the modification results in the introduction of an extra amine group, which increases the ionization efficiency of the peptide, especially in relation to the phosphorylated peptide.

In order to confirm the applicability of our enrichment strategy, we composed a mixture of synthetic nonphosphorylated peptides[42,43] to which the serine-phosphorylated octapeptide **2a** was added (~1 nmol each; see Figure 2A and Table 1).

Table 1. Components of the peptide mixture used for phosphopeptide enrichment experiments.

Peptide sequence	*m*/*z*
Ac-GFEApSLFK-NH_2	1019.4
H-SNNFGALLGS-NH_2	978.5
Ac-VHAEFLKA-NH_2	955.5
Ac-ARAEQLAQEA-NH_2	1127.6
Ac-KLB[a]EII-NH_2	769.5
H-GFEATLFK-NH_2	911.5
H-SNNFGAILSS-NH_2	1008.5
Ac-LAQEAHKNAKLB[a]EII-NH_2 ($E_4 \rightarrow K_7$ lactam)	1714.0
Ac-EB[a]ARAEQLAQEAHK-NH_2 ($E_{11} \rightarrow K_{14}$ lactam)	1616.8
Ac-REVLEB[a]ARAEQLAQEAHKNRKLB[a]EII-NH_2	3111.8

[a] B = L-norleucine.

The peptide mixture was subjected to the optimized β-elimination/EDT addition protocol. After evaporation of the volatiles, the residue was redissolved in MeOH (90 μL) and incubated at 45 °C for 2 h with probe molecule **10** (5–50 equiv). Subsequently, the mixture was incubated with immobilized avidin for 1 hour, and the beads were then washed with buffer and

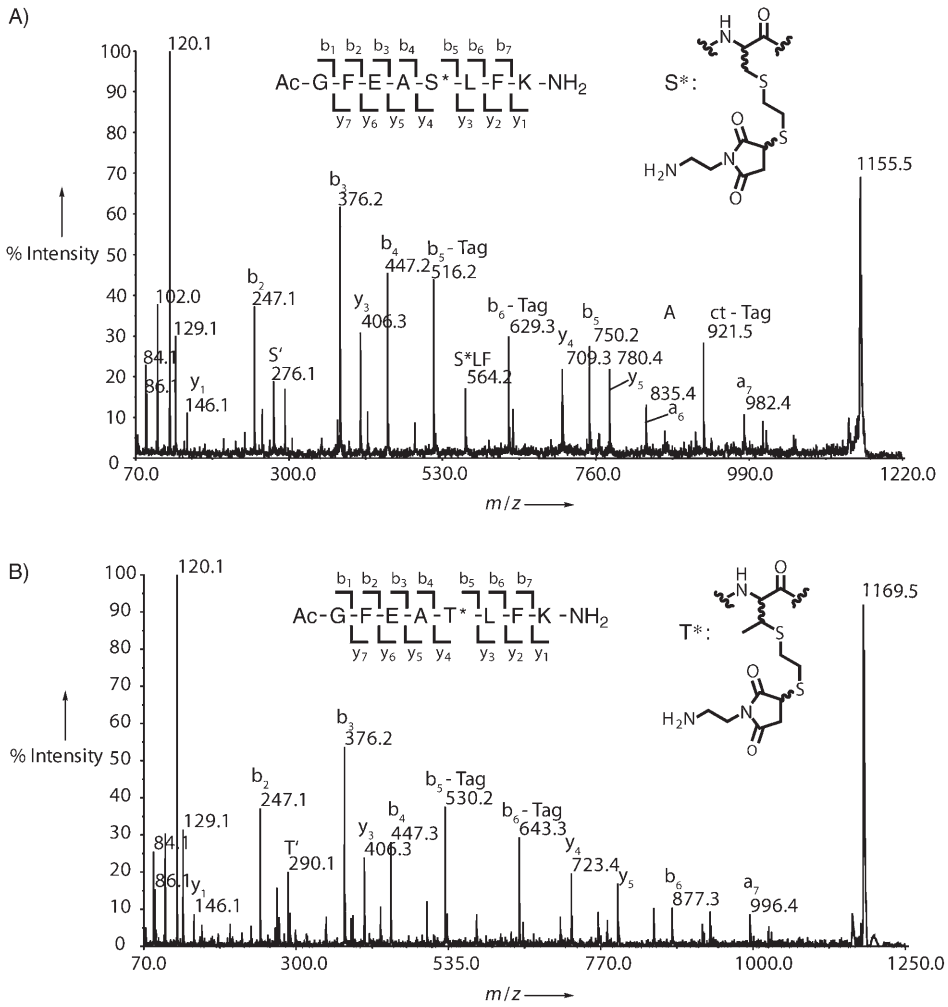


Figure 1. Mass spectrometric fragmentation (MS/MS) spectra of the modified phosphopeptides **2a** and **2b**. Characteristic b- and y-type ions that were used for sequence tagging of the peptide are indicated. A) MS/MS spectrum of the modified serine-phosphorylated peptide (*m/z* 1155.5). The structure of the modified phosphoserine moiety is shown. The peak marked S′ indicates the immonium ion of the modified amino acid residue S*. The peaks marked "−Tag" (at *m/z* 516.2, 629.3, and 921.5) indicate the corresponding fragments that have lost the complete modification (the probe residue including the EDT moiety) as a result of β-elimination during MS/MS. B) MS/MS spectrum of the modified threonine-phosphorylated peptide (*m/z* 1169.5). The structure of the modified phosphothreonine moiety is shown. The peak marked T′ indicates the immonium ion of the modified amino acid residue T*. The peaks marked "−Tag" (at *m/z* 530.2, 643.3, and 939.5) indicate the corresponding fragments that have lost the complete modification (the probe residue including the EDT moiety) as a result of β-elimination during MS/MS.

treated with TFA to cleave the Wang-type linker. After removal of TFA, the mixture was analyzed by MALDI-TOF mass spectrometry. The mass spectrum of the enriched fraction is shown in Figure 2B. The most abundant peak, at *m/z* 1155.5, corresponds to the modified phosphopeptide. The presence of other compounds (peptides) in the enriched fraction might be caused by unspecific binding of particular peptides either to avidin or to agarose on which the avidin was immobilized. These peaks however, can be used to calculate a semiquantitative amount of enrichment: the intensity ratio between the synthetic peptide with an *m/z* value of 1127.6 and the phosphopeptide (*m/z* 1019.4, marked with P in Figure 2A) before enrichment is 10, while it is 0.11 after enrichment, yielding a semiquantitative enrichment factor of around 100.

Together with the sequence tagging and identification of the former site of phosphorylation, as shown in Figure 1, these results confirm the validity of our methodology for modification, enrichment, and analysis of serine and threonine phosphorylated peptides.

Conclusion

The analysis of post-translational modifications on proteins in complex mixtures, especially that of protein phosphorylation, requires methods that allow specific enrichment of peptides that carry this type of modification. This is because mass spectrometry, routinely used for these purposes, has some disadvantages that hamper the investigation of this modification. The extra negative charge, for example, lowers the ionization efficiency of phosphorylated peptides in the positive-ion mode, and MS/MS collision-induced fragmentation of these ions mainly results in loss of the labile phosphate group, obstructing further sequence analysis.

In order to improve the analysis of phosphorylated peptides, we have developed a protocol for the selective β-elimination and subsequent EDT modification of serine- and threonine-phosphorylated peptides using three different test systems with different degrees of complexity. In our test systems, this newly developed protocol proved more effective than previously described methods.

It was found that β-elimination of phosphorylated Ser/Thr residues took place without concomitant β-elimination of unphosphorylated Ser/Thr residues. So far, no reliable procedure for selective β-elimination of phosphorylated Ser/Thr residues in the presence of glycosylated Ser/Thr residues has been described. Selective β-elimination of phosphate in the presence of other modified (e.g., glycosylated) Ser/Thr residues is currently the subject of investigation, and preliminary results are promising.

Furthermore, selective profiling of Ser/Thr phosphorylation or glycosylation can be facilitated by pretreatment of the peptide or protein mixture with an appropriate glycosidase or phosphatase, respectively.

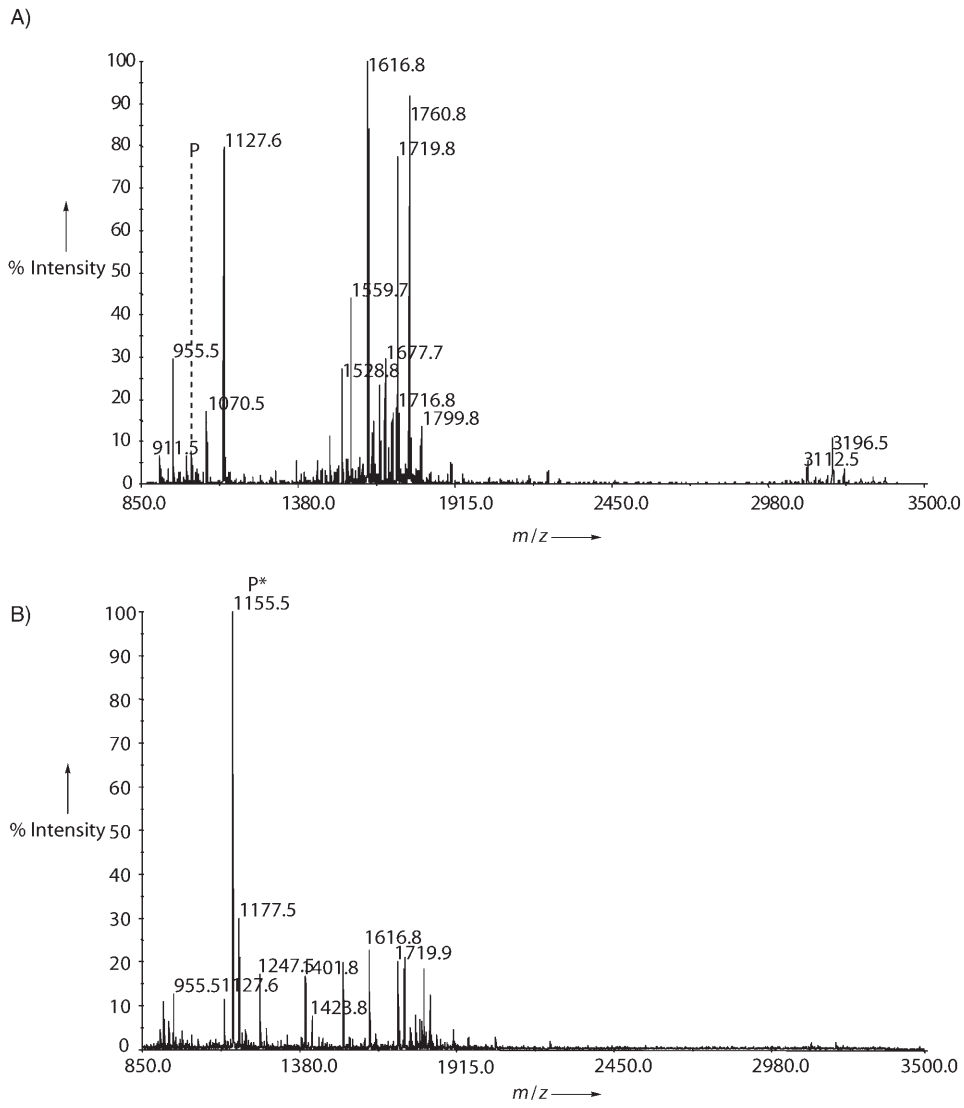


Figure 2. A) MALDI-TOF mass spectrum of the starting mixture of peptides used to mimic a tryptic digest to test the chemical modification procedure. The phosphopeptide (**2a**) at *m/z* 1019.4 is indicated by a dashed line and the letter P. B) MALDI-TOF mass spectrum of the supernatant obtained after affinity enrichment and cleavage of the Wang-type linker. The formerly phosphorylated species is indicated by P* ($[M+H]^+$, *m/z* 1155.5, $[M+Na]^+$, *m/z* 1177.5).

In addition, we have developed a multifunctional molecular probe featuring a biotin affinity label, an acid-labile linker, a site for the optional incorporation of stable isotopes, and an electrophilic reactive group. This probe molecule selectively allows further modification of originally phosphorylated serine and threonine residues, as well as affinity-based purification of modified phosphopeptides from mixtures of peptides. Modified peptides can be released from the affinity purification material by cleavage of the acid-labile linker, and the remaining, compact adduct allows identification of the peptide sequence, as well as of the phosphorylation site. Incorporation of an acid-labile linker ensures quantitative release of bound peptides from the affinity purification material unlike previously reported biotin-based molecular probes. In addition, MS/MS spectra of the modified peptides are more straightforward when the adducts do not contain biotin, and sequencing of the peptide and assignment of the phosphorylation site is simpler.

The work described here offers an approach to better analysis of phosphorylated peptides. It not only enables affinity enrichment of originally phosphorylated peptides, but it also improves further analysis of these peptides by introduction of a stable group, which, moreover, provides improved ionization due to the presence of an additional amino group.

The introduction of stable isotopes could offer potential for quantitative phosphoproteomic studies that not only give insight into the pool of phosphorylated proteins, but also reveal some of the dynamics of this modification over time or between different cellular states.

In summary, the pursuit of knowledge on the down-scaling of chemical reactions and incorporation of stable isotopes for relative quantitation of phosphorylation levels is a prerequisite for further improvement of the field of chemistry-based phosphoproteomics, allowing the analysis of phosphorylated peptides in complex biological samples.

Experimental Section

General: All solvents were distilled prior to use or were HPLC grade. Anhydrous solvents were obtained by storing the solvents over activated 4 Å molecular sieves. Tentagel S RAM resin was purchased from Rapp Polymere (Tübingen, Germany). Fmoc amino acids were purchased from Advanced ChemTech (Louisville, KY, USA), Alexis (San Diego, CA, USA), or Novabiochem (Läufelingen, Germany). Avidin immobilized on 6% agarose was purchased from Sigma. All other reagents were purchased from Fluka, Sigma–Aldrich, and Acros and were used without further purification. Solid-phase peptide synthesis was performed on an Applied Biosystems 433 A peptide synthesizer. TLC plates used were Merck 60 F_{254} silica gel on glass. Visualization was achieved with UV light when applicable, or by staining with ninhydrin solution in *n*-butanol (0.2%). NMR spectra were recorded on a Varian Inova 300 MHz spectrometer. Chemical shifts are reported in ppm downfield, relative to tetramethylsilane. ESI-MS spectra were obtained in the positive-ion mode on a Shimadzu instrument. MALDI-TOF spectra were acquired in reflectron positive-ion mode on an Applied Biosystems Voyager DE-STR instrument or an Applied Biosystems 4700 Proteomics Analyzer with α-cyano-4-hydroxycinnamic acid as the matrix. MS/MS spectra were acquired on an Applied Biosystems 4700 Proteomics Analyzer.

Synthesis of peptides: Tripeptides **1a** and **1b** were synthesized by a standard *tert*-butyloxycarbonyl protection strategy with BOP as coupling agent and Et_3N as base in CH_2Cl_2, from *N*-Boc-*O*-benzyl-serine or -threonine. Treatment with HCl in Et_2O was used for Boc cleavage. *N*-Termini were acetylated by treatment with AcOSu. After hydrogenolysis of the benzyl ethers, the resulting free alcohols were phosphorylated as described earlier.[38] Because of their more straightforward NMR spectra, the bis(*p*-chlorobenzyl) phosphotriesters of **1a** and **1b** (**1aa** and **1bb**, respectively) were used for analysis.

Ac-Phe-Ser[*O*-P(O)(*p*-ClC$_6$H$_4$CH$_2$O)$_2$]-Gly-NHMe (1aa): ^{1}H NMR (300 MHz, CD_3OD): $\delta = 2.00$ (s, 3H), 2.84 (s, 3H), 3.02 (m, 1H), 3.28 (m, 1H), 3.85 (d, 1H), 4.01 (d, 1H), 4.49 (m, 1H), 4.75 (m, 1H), 5.17 (d, $J = 8.5$ Hz, 2H), 7.25–7.45 ppm (m, 13H); ^{13}C NMR (75 MHz, CD_3OD): $\delta = 25.2$, 29.2, 41.3, 46.3, 59.2, 70.4, 73.0, 132.2, 132.6, 133.1, 133.6, 138.4, 138.5, 138.6, 141.1, 173.4, 174.5, 176.4, 177.3 ppm.

Ac-Phe-Thr[*O*-P(O)(*p*-ClC$_6$H$_4$CH$_2$O)$_2$]–Gly-NHMe (1bb): ^{1}H NMR (300 MHz, CD_3OD): $\delta = 1.36$ (d, $J = 6.3$ Hz, 3H), 1.90 (s, 3H), 2.68 (s, 3H), 2.94 (dd, $J = 14.1$, 9.1 Hz, 1H), 3.17 (dd, $J = 14.1$, 6.3 Hz, 1H), 3.69 (d, $J = 16.7$ Hz, 1H), 3.83 (d, $J = 16.8$ Hz, 1H), 4.75 (m, 1H), 5.01 (m, 2H), 5.09 (m, 1H), 7.19–7.37 ppm (m, 13H); ^{13}C NMR (75 MHz, CD_3OD): $\delta = 16.7$, 21.2, 25.2, 37.2, 42.3, 55.1, 57.2, 68.8, 75.8, 126.7, 128.4, 128.6, 129.2, 129.5, 129.6, 134.4, 134.7, 137.2, 169.6, 170.4, 172.2, 173.5 ppm.

Octapeptides 2a and 2b: Octapeptides **2a** and **2b** were synthesized on an Applied Biosystems 433 A peptide synthesizer with (*p*-{(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl}-phenoxyacetic acid)-crosslinked polystyrene as the starting resin (loading 0.3 mmol g^{-1}) in order to obtain a C-amidated sequence. *tert*-Butyloxycarbonyl (Boc) side-chain protecting groups were used for lysine and *tert*-butyl for glutamic acid. The *N*-α-amino acids were activated in situ by the standard 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) procedure. Fmoc deprotection was carried out with piperidine in DMF (20%). Final acetylation was carried out by shaking the resin-bound peptide overnight with *N*-hydroxysuccinimidyl acetate (5 equiv.) in DMF. Phosphorylation was achieved by shaking the resin with reagent **28a** (for **2a**) or reagent **28b** (for **2b**) (10 equiv.) and 1*H*-tetrazole (50 equiv.) in dry *N*,*N*-dimethyl acetamide (DMA) for 24 h, followed by addition of a solution of *tert*-butyl hydroperoxide in H_2O (70%, 15 equiv.). After 30 min, the solvent was removed by suction filtration and the resin was successively washed several times with DMA, CH_2Cl_2, and MeOH. Cleavage of the peptide from the resin was accomplished by treatment of the peptidyl resin with a mixture of TFA and scavengers (TFA/H_2O/EDT/TIS 88:8:3:1 *v*/*v*/*v*/*v*). The crude peptide was precipitated from cold *tert*-butyl methyl ether and hexane (50:50), redissolved in H_2O/*tert*-butanol (50:50), and lyophilized.

Synthesis of probe molecules: For experimental data and characterization of compounds **6** and **13–18**, see the Supporting Information.

Methyl 4-(2-{[(benzyloxy)carbonyl]amino}ethoxy)benzoate (19): Benzyl chloroformate (2.4 g, 20 mmol) was added dropwise to an ice-cooled solution of 2-bromoethylamine·HBr (4.74 g, 22 mmol) and Et_3N (4.4 g, 44 mmol) in CH_2Cl_2 (60 mL). After the addition, the cooling bath was removed and the solution was allowed to reach room temperature. Stirring was continued for 2 h, followed by evaporation of volatile components under reduced pressure. The remaining oil was mixed with EtOAc (200 mL). The organic layer was washed with aq. HCl (1 N, 100 mL) and brine (100 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo to give crude *N*-Cbz-2-bromoethylamine as a colorless oil, which was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 3.44 (t, $J = 5.4$ Hz, 2H), 3.65 (q, $J = 5.4$ Hz, 2H), 5.12 (s, 2H,), 5.22 (t, $J = 5.4$ Hz, 1H), 7.24–7.41 ppm (m, 5H). A solution of the crude *N*-Cbz-2-bromoethylamine (3.9 g, ~15 mmol) and methyl 4-hydroxybenzoate (**12**, 2.28 g, 15 mmol) in MeOH (10 mL) was adsorbed on solid anhydrous K_2CO_3 (10.4 g, 75 mmol) by evaporation of the solvent under reduced pressure. The resulting cake was irradiated in a kitchen microwave apparatus (5×1 min with intermediate cooling). The crude product was obtained by washing of the cake with EtOAc/MeOH (9:1, 150 mL) and evaporation of the solvent. Product **19** was isolated after chromatography on silica gel (hexane/EtOAc 1:1) as a white solid (yield: 4.02 g, 12.2 mmol, 83%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.63$ (q, $J = 5.4$ Hz, 2H), 3.88 (s, 3H), 4.09 (t, $J = 5.4$ Hz, 2H), 5.11 (s, 2H), 5.26 (t, $J = 5.4$ Hz, 2H), 6.89 (d, $J =$ 8.7 Hz, 2H), 7.33 (m, 5H), 7.98 ppm (d, $J = 8.7$ Hz, 2H); MS (ESI): *m*/*z* 330.3 $[M+H]^+$, 352.4 $[M+Na]^+$.

Benzyl 2-[4-(hydroxymethyl)phenoxy]ethylcarbamate (20): Methyl ester **19** (4.0 g, 12.1 mmol) was dissolved in a mixture of THF and Et_2O (60 mL) and was cooled to 0 °C under nitrogen atmosphere with an ice bath. $LiAlH_4$ (740 mg, 20 mmol) was added in three portions over a period of 60 min. The reaction mixture was stirred for 6 h at 0 °C and was then carefully quenched by the dropwise addition of water (5 mL). The solid precipitate was filtered off and washed with THF (3×20 mL). Evaporation of the solvent yielded crude **20**, which was purified by silica gel chromatography (EtOAc) (yield: 3.42 g, 11.36 mmol, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.1$ (br s, 1H), 3.61 (q, $J = 5.1$ Hz, 2H), 4.04 (t, $J = 5.4$ Hz, 2H), 4.6 (s, 2H), 5.12 (s, 2H), 5.24 (t, $J = 5.4$ Hz, 2H), 6.83 (d, $J =$ 8.4 Hz, 2H), 7.28 (d, $J = 8.4$ Hz, 2H), 7.3–7.42 ppm (m, 5H); MS (ESI): *m*/*z* 302.1 $[M+H]^+$.

4-(2-Aminoethoxy)benzyl alcohol (21): *N*-Cbz-protected alcohol **20** (3.0 g, 10 mmol) was dissolved in MeOH (20 mL). After flushing with N_2 and addition of Pd on C (10%, 210 mg, 2 mol%), the reaction mixture was placed under an atmosphere of H_2 and stirred for 8 h at room temperature. After removal of excess hydrogen by flushing with N_2, the catalyst was removed by filtration through a short path of Celite and the solvent was removed under reduced pressure, yielding **21** as a colorless solid that was used in the next step without further purification (yield: 494 mg, 2.94 mmol, 98%). ^{1}H NMR (300 MHz, CD_3OD): $\delta =$ 2.84–3.09 (m, 2H), 3.97 (t, $J =$ 5.1 Hz), 4.53 (s, 2H), 6.92 (d, $J = 8.7$ Hz), 7.27 ppm (d, $J = 8.7$ Hz).

***tert*-Butyl 2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethylcarbamate (22)**: Monoprotected *N*-Boc-ethylenediamine (3.2 g, 20 mmol) and Et_3N (2.0 g, 20 mmol) were dissolved in Et_2O (40 mL) at 0 °C. A solution of maleic anhydride (1.96 g, 20 mmol) in Et_2O (40 mL) was added dropwise, and the reaction mixture was stirred for 4 h while being allowed to reach room temperature. The precipitated crude intermediate triethylammonium salt was filtered off and dissolved in CH_2Cl_2 (100 mL). Additional Et_3N (4.0 g, 40 mmol) and BOP (8.84 g, 20 mmol) were added and stirring was continued for two hours. CH_2Cl_2 was evaporated under reduced pressure and the dark brown residue was redissolved in EtOAc (200 mL). The organic layer was washed with aq. HCl (1 N, 2×100 mL), sat. aq. $NaHCO_3$ (100 mL), and brine (100 mL). After drying over Na_2SO_4, the organic layer was concentrated under reduced pressure. The dark brown, oily residue was chromatographed on silica gel (hexane/EtOAc 1:1), yielding **22** as a white, crystalline solid (yield: 1.83 g, 7.6 mmol, 38%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.4$ (s, 9H), 3.34 (q, $J = 5.7$ Hz, 2H), 3.64 (t, $J = 5.7$ Hz, 2H), 5.01 (t, $J = 5.7$ Hz, 1H), 6.74 ppm (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 28.12$ (s, Boc), 37.8, 39.1, 79.3,

134.0, 155.9, 170.8 ppm; MS (ESI): *m/z* 140.3 $[(M-\text{Boc})+\text{H}]^+$, 240.4 $[M+\text{H}]^+$.

1-(2-Aminoethyl)-1*H*-pyrrole-2,5-dione HCl salt (23): *N*-Boc-protected maleimide **22** (1 g, 4.17 mmol) was dissolved in CH_2Cl_2 (2 mL). A saturated solution of hydrogen chloride in Et_2O (5 mL) was added. After the mixture had been stirred for 30 min, the precipitated hydrochloride salt was filtered off and washed with Et_2O (2×5 mL). Product **23** was obtained as a white powder (yield: 702 mg, 4.0 mmol, 96%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.12$ (t, $J =$ 5.7 Hz, 2H), 3.77 (t, $J = 5.7$ Hz, 2H), 6.84 ppm (s, 2H); MS (ESI): *m/z* 140.3 $[M+\text{H}]^+$.

4-Nitrophenyl 2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethylcarbamate (24): A solution of 4-nitrophenyl chloroformate (800 mg, 4 mmol) in CH_2Cl_2 (5 mL) was cooled with an ice/salt bath, and powdered **23** was added, followed by dropwise addition of Et_3N (800 mg, 8 mmol). After the mixture had been stirred for two hours, the solvent was removed and the residue was redissolved in EtOAc (100 mL). The organic layer was washed successively with aq. HCl (1 N, 2×25 mL) and brine (25 mL). The yellow residue was chromatographed on silica gel (hexane/EtOAc 2:1) and the target product **24** was obtained as a pink solid (yield: 455 mg, 1.5 mmol, 37%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.42$–3.61 (m, 2H), 3.76–3.84 (m, 2H), 5.6 (br s, 1H), 6.75 (s, 2H), 7.29 (d, $J = 12$ Hz, 2H), 8.23 ppm (d, $J = 12$ Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 36.9$, 37.6, 39.8, 40.8, 122.4, 125.4, 134.6, 145.1, 153.7, 156.0, 171.1 ppm; MS (ESI): *m/z* 306.5 $[M+\text{H}]^+$, 328.4 $[M+\text{Na}]^+$.

***N*-{2-[4-(Hydroxymethyl)phenoxy]ethyl}-5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (27)**: Amine **21** (184 mg, 1.1 mmol) was dissolved in DMF (5 mL). The *N*-hydroxysuccinimidyl ester of biotin (**26**, 330 mg, 1 mmol) was added, and the reaction mixture was stirred for 1 hour at room temperature. The solvent was evaporated under reduced pressure and the resulting solid was washed with MeCN (4×2 mL), yielding **27** as a colorless solid (322 mg, 0.85 mmol, 85%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.18$–1.64 (m, 6H), 2.08 (t, $J = 7.2$ Hz, 2H), 2.44–2.61 (m, 1H), 2.79 (dd, $J = 12.3$, 4.8 Hz, 1H), 2.98–3.16 (m, 1H), 3.38 (q, $J = 5$ .7 Hz, 2H), 3.94 (t, $J = 5.7$ Hz, 2H), 4.06–4.11 (m, 1H), 4.22–4.34 (m, 1H), 4.4 (s, 2H), 4.8–5.2 (br s, 1H), 6.37 (s, 1H), 6.44 (s, 1H), 6.87 (d, $J = 8.71$ Hz, 2H), 7.21 (d, $J = 8.7$ Hz, 2H), 8.06 ppm (t, $J = 5.7$ Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): $\delta = 24.9$, 25.7, 28.5, 33.6, 39.0, 39.9, 55.8, 60.4, 62.1, 63.7, 66.4, 114.3, 128.5, 133.9, 158.4, 164.9, 175.3 ppm; MS (ESI): *m/z* 376.1 $[M-\text{OH}]^+$, 394.5 $[M+\text{H}]^+$, 416 $[M+\text{Na}]^+$.

4-(2-{[5-(2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoyl]amino}ethoxy)-benzyl 2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl carbamate (10): Benzylic alcohol **27** (190 mg, 0.5 mmol) and 4-nitrophenyl carbonate **24** (140 mL, 0.6 mmol) were dissolved in dry DMF. Et_3N (100 mg, 1 mmol) was added, and the mixture was stirred for 48 h at room temperature under a nitrogen atmosphere. The solvent was evaporated under reduced pressure, and the residue was chromatographed on silica gel (CH_2Cl_2/MeOH 90:10). Compound **10** was obtained as a white solid (120 mg, 0.22 mmol, 37%). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 1.42$ (q, $J =$ 7.2 Hz), 1.47–1.92 (m, 4H), 2.24 (t, $J = 7.2$ Hz, 2H), 2.66 (d, $J =$ 10.8 Hz, 1H), 2.86 (dd, $J = 12.6$, 5.4 Hz, 1H), 3.05–3.18 (m, 1H), 3.24–3.41 (m, 4H), 3.52–3.75 (m, 4H), 4.04 (t, $J = 5.4$ Hz), 4.19 (dd, $J = 7.8$, 5.2 Hz, 1H), 4.42 (dd, $J = 7.8$, 5.2 Hz, 1H), 4.95 (s, 2H), 6.77 (s, 2H), 6.91 (d, $J = 5.7$ Hz, 2H), 7.25 ppm (d, $J = 5.7$ Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 25.6$, 28.2, 28.4, 35.5, 37.5, 39.0, 39.9, 39.9, 55.8, 60.4, 61.9, 66.0, 66.4, 114.3, 129.5, 134.2, 158.5, 165.8, 171.4, 175.2 ppm; MS (ESI): *m/z* 376.1, 559.4 $[M+\text{H}]^+$, 581.2 $[M+\text{Na}]^+$.

Modification of phosphopeptides: A solution of a mixture of peptides (10 μL, containing 0.1–1 nmol of phosphopeptide in 10–50% aq. MeOH), MeOH (80 μL), and sat. aq. $Ba(OH)_2$ (0.15 N, 3 μL, 450 nmol) in a 500 μL Eppendorf vial was vortexed and shaken in an Eppendorf Thermomixer apparatus (800 rpm) for 3 h at 45 °C. Aq. H_2SO_4 (0.30 N, 1.5 μL, 450 nmol) was then added, and the mixture was vortexed and centrifuged (4 min. at 12000 rpm) to precipitate $BaSO_4$ and $Ba_3(PO_4)_2$. The supernatant was transferred to a new 500 μL Eppendorf vial, and EDT (18 μmol) and Et_3N (11 μmol) were added. The resulting mixture was vortexed and shaken (800 rpm) for 48 h at 45 °C. The Eppendorf vial was then briefly centrifuged to pull down any droplets sticking to the cap of the vial. An aqueous solution of TCEP·HCl (tris(carboxyethyl)phosphine HCl salt, 10 μL, 1 mM, 10 nmol) was added and the vial was vortexed and briefly centrifuged again. All volatile components were evaporated by placing the open vial in the Thermomixer apparatus at 45 °C with gentle shaking (300 rpm) under a continuous argon stream. MeOH (100 μL) was added to the residue, and the vial was vortexed and centrifuged (3 min at 12000 rpm). The supernatant was transferred to a new 500 μL Eppendorf vial and the volatile components were evaporated by gentle shaking (300 rpm) of the open vial in the Thermomixer apparatus at 45 °C under a continuous argon stream. After evaporation, additional MeOH (100 μL) was added and evaporated again; this was repeated once. The residue was dissolved in MeOH (90 μL) and a 5 mM solution of **10** in MeOH (10 μL, 50 nmol) and Et_3N (100 mM in MeOH, 10 μL) was added. The mixture was vortexed and shaken (800 rpm) for 3 h at 45 °C. Unreacted maleimide groups were capped by addition of β-mercaptoethanol (1 μL). Again, volatile components were evaporated by gentle shaking (300 rpm) of the open vial in the Thermomixer apparatus at 45 °C under a continuous argon stream. The residue was redissolved in phosphate buffer (pH 7.4, 100 mM) containing NaCl (200 mM, 30 μL), and a slurry of avidin immobilized on 6% agarose (10 μL) was added. The mixture was gently shaken for 2 h in an Eppendorf Thermomixer apparatus (1000 rpm, 25 °C). The supernatant was removed and the beads were washed with buffer (5×30 μL). The beads were then shaken (1000 rpm, 25 °C) for 1 h with TFA (95%, 30 μL) and the supernatant containing the modified peptide(s) was transferred to a new 500 μL Eppendorf vial. Finally, volatile components were evaporated by gentle shaking (300 rpm) of the open vial in the Thermomixer apparatus at 45 °C under a continuous argon stream. The residue was then analyzed by MALDI-TOF MS.

Keywords: beta-elimination · chemical proteomics · Michael addition · molecular probes · phosphorylation

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